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Induction of Metamorphosis in the Sea Urchin *Holopneustes purpurascens* by a Metabolite Complex From the Algal Host *Delisea pulchra*

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Abstract. Most benthic invertebrates have complex life cycles with planktonic larvae that return to the substratum to settle and metamorphose into a benthic stage. Although naturally produced chemical cues have long been thought to be important for the settlement or metamorphosis of invertebrate larvae, few ecologically relevant chemical cues have been clearly identified. The marine echinoid *Holopneustes purpurascens* has a complex life cycle, with a planktonic, nonfeeding dispersive larva that metamorphoses into a benthic stage that lives in the canopy of subtidal benthic algae such as the red alga *Delisea pulchra* and the kelp *Ecklonia radiata*. Recently recruited juveniles are found primarily on *D. pulchra*, and we hypothesized that this was in response to a chemical cue produced by this alga. Competent larvae metamorphosed in the presence of *D. pulchra*, or seawater surrounding this alga, but not in response to the presence of *E. radiata* or its extracts. A cue for metamorphosis was isolated and characterized from *D. pulchra* and found to be a water-soluble complex of the sugar floridoside and isethionic acid in a 1:1 molar ratio. The floridoside–isethionic acid complex also triggered settlement in *H. purpurascens*; however, this response was less specific than metamorphosis and was reversible. Larvae of *H. purpurascens* also metamorphosed in the presence of several other species of red, but not brown or green, algae from their habitat. Floridoside is found only in red algae, suggesting that the floridoside–isethionic acid complex may be acting as a cue for metamorphosis in other red algae as well as in *D. pulchra*.

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Introduction

A fundamental problem in marine biology and ecology is understanding the demography of organisms with complex life histories in which planktonic and benthic stages occur in spatially distinct habitats (Pechenik, 1999). In particular, the question of how a planktonic larva that has dispersed through the water column since fertilization finds an appropriate benthic habitat for metamorphosis and the resumption of the benthic stage has been a central theme in the study of marine invertebrates for over 40 years (Thorson, 1950; Butman, 1987; Roughgarden *et al.*, 1988; Pawlik, 1992; McEdwards, 1995).

The current view of this issue is that successful recruitment of larvae back to the benthos is the result of a mixture of processes. Hydrodynamic processes are thought to dominate at larger scales, by controlling whether larvae are deposited at a suitable area when competent. Environmental cues are thought to become progressively more important at smaller spatial scales, such as finding a specific site or habitat within the area (Scheltema, 1986; Young and Chia, 1987; Butman *et al.*, 1988). These environmental cues may be physical, biological, or chemical (Svane and Young, 1989; Pawlik, 1992; McEdwards, 1995), but chemical cues in particular have received considerable attention in the literature. For many species, chemical cues associated with substrata appear to be of primary importance to competent larvae just prior to settling and metamorphosing (Burke, 1983; Pawlik and Hadfield, 1990; Pawlik, 1992).

Although there has been considerable screening of chemical compounds as potential cues that induce settlement and metamorphosis of marine invertebrates, few studies have definitively identified (characterized) chemical inducers for

these processes. Moreover, the ecological relevance of many inducers is not always clear. The majority of research in this area has focused on the effects of exogenous (to the target organism) metabolites, *e.g.* "artificial" inducers *sensu* Pawlik (1992). Examples include γ-aminobutyric acid (GABA) (Morse *et al.*, 1979), L-β-3,4-dihydroxyphenylalanine (L-DOPA) (Coon and Bonar, 1987), and epinephrine (Coon *et al.*, 1986), which have not been shown to occur naturally in the system being examined but are thought to act in a manner similar to those that do. The few studies that have identified putative naturally occurring inducers have either not characterized the compound or lacked data to support their ecological relevance (*e.g.*, Yvin *et al.*, 1985); that is, the presence of the inducer did not relate to the recruitment pattern of the organism (although see Tsukamato *et al.*, 1999).

The sea urchin *Holopneustes purpurascens* is an endemic Australian echinoid that lives in shallow subtidal waters. This urchin is atypical of echinoids because it inhabits the canopy of its two main host plants, *Delisea pulchra* and *Ecklonia radiata*, using the algae that it consumes as a habitat as well as a source of food (Steinberg, 1995). Thus, its distribution across habitats is tightly coupled with restrictions in diet breadth. Since both species of host algae are rich in secondary metabolites (Steinberg, 1989; van Altena and Steinberg, 1992; de Nys *et al.*, 1993), it was hypothesized that competent larvae may recruit to these hosts in response to a specific chemical settlement or metamorphosis cue.

In this study, we assess the response of competent larvae of *H. purpurascens* to potential chemical inducers from its host algae. Since such chemical cues can be either aggregative (released by conspecifics: Crisp, 1974; Burke, 1986) or associative (released by non-conspecifics such as host plants: Pawlik, 1992), we first determined whether *H. purpurascens* was settling or metamorphosing in response to cues associated with newly recruited conspecifics. The response of *H. purpurascens* larvae to algae (and their metabolites) associated with the urchin's habitat was then assessed. Where there was an indication of a potential positive inducer, this cue was isolated and identified through a series of bioassay-guided fractionations. Once the inducer had been isolated and identified, conditioned seawater was collected *in situ* and also tested to determine whether an inducer was present in the field. Finally, other algae in the habitat were tested for their ability to induce metamorphosis of larvae of *H. purpurascens*.

Materials and Methods

Study site and animal

This study was done in sublittoral habitats (3–5 m depth) at Bare Island (33° 59′ 38″ S, 151° 14′ 00″ E) at the north head of Botany Bay, Sydney, Australia. Bare Island is a mosaic of habitats typical of shallow subtidal areas in temperate southeastern Australia. Adult *Holopneustes purpurascens* (Temnopleuridae: Echinodermata) at this site are principally found on laminae of the kelp *Ecklonia radiata* (Laminariales: Phaeophyta) and in the fronds of the red foliose alga *Delisea pulchra* (Bonnemaisonales: Rhodophyta). Steinberg (1995) further describes some aspects of the ecology of *H. purpurascens* in these habitats.

Size distribution and abundance of Holopneustes purpurascens *on host plants*

Size distributions of *H. purpurascens* on individual plants of *D. pulchra* and *E. radiata* at Bare Island were assessed by measuring the maximum test diameter to the nearest millimeter of all *H. purpurascens* present *in situ* within an area of 10 m^2. Sampling was done during March (autumn) 1996 and repeated in September (spring) 1996; the data from both seasons were pooled. The size of urchins between the two host plants was compared with a two-tailed unpaired Student's *t* test.

The abundance of *H. purpurascens* on *D. pulchra* and *E. radiata* was also measured at Bare Island during March (autumn) 1996. Abundance was measured as the number of urchins per plant. The data was first tested for heterogeneity of variance using a Cochran's test (Winer, 1971) before analyzing differences in abundance between host plants with a one-factor analysis of variance (ANOVA).

Culture of larvae

All larval assays of *H. purpurascens* were done with larvae reared in the laboratory at the University of New South Wales, Australia. Larval cultures were established using gametes from several male and female urchins, obtained by intracoelomic injections with 3–5 ml (depending on their size) of 0.5 *M* KCl to induce spawning (Pennington, 1985; Levitan *et al.*, 1992). Viable eggs were fertilized immediately and washed twice (~15 min between adding sperm and washing) in sterile filtered seawater. This seawater had passed through a 0.2-μm filter, been autoclaved overnight, and had 36.5 mg l^{-1} streptomycin and 21.9 mg l^{-1} penicillin added to it. Fertilization success was determined as the percentage of eggs that had raised vitelline membranes. In all cases, at least 95% of the eggs were fertilized. Fertilized eggs were maintained in 2-l glass beakers at a maximum density of 100 eggs ml^{-1} until hatching, when this density was reduced to about 5 larvae ml^{-1}. Throughout their development, cultures were kept at 18°C in a 12-h light/12-h dark regime with a slow bubbling of filtered air. The seawater was changed every 24 h, and abnormal larvae (as determined by microscopy) were removed. Larvae reached competency (recognized by the presence of five well-developed tube feet) within 6 days,

and those that had just developed competency were used in assays.

Initially, both settlement and metamorphosis were assessed. However, settlement for *H. purpurascens* is a reversible process in which the larvae cease swimming, cling to the substratum using their tube feet, and appraise the suitability of the substrate for metamorphosis. Metamorphosis in *H. purpurascens* is an irreversible developmental process in which the larvae change from bilateral to radial symmetry. Thus, metamorphosis is a clear transition from the planktonic stage to the benthic stage in the urchin's life history. Therefore, following initial experiments, assays focused on metamorphosis as an end point. In all experiments, percent metamorphosis was determined after a designated time interval (usually 24 h), and calculated as the number of larvae metamorphosed relative to the total number of replicate larvae for that treatment. Following experiments demonstrating that larval metamorphosis was not aggregative (below), all assays used one larva per replicate petri dish (40-mm diameter) in 5 ml of sterile filtered seawater (to maintain replicate dishes while minimizing the number of larvae needed).

Larval response to conspecifics

To determine whether settlement or metamorphosis of *H. purpurascens* was aggregative, larvae were exposed to treatments containing newly metamorphosed (7 days old) conspecifics in two densities and water that had been conditioned by adult conspecifics. Newly metamorphosed conspecifics were reared in the laboratory (see section above) and metamorphosed in bulk in the presence of pieces of *D. pulchra* 7 days prior to the start of the experiment. These individuals were then transferred to two 2-l glass beakers containing sterile filtered seawater, which was changed daily, and a continuous flow of filtered air. Metamorphosed individuals were identified on the day of the experiment by their change in shape, the presence of chromatophores, the start of spine development, and their active behavior. At the start of the experiment, one competent larva was added to treatments containing either one or five of these newly metamorphosed conspecifics in glass petri dishes with 5 ml of sterile filtered seawater.

Water conditioned by adult *H. purpurascens* was prepared by collecting 16 healthy mature urchins (20–45 mm diameter) from Bare Island and, in the laboratory, immersing each individual in 500 ml sterile filtered seawater for 3 h. Five millilitres of this conditioned seawater (containing no particulate matter) was then removed from each of the 500-ml replicates (~76 l in total) and added to a separate petri dish.

One competent larva was added to each replicate petri dish for each of the three treatments (the two densities of new recruits and adult conditioned water) and replicate controls of sterile filtered seawater. The controls (sterile filtered seawater) were included as a measure of settlement or metamorphosis in the absence of a cue. The percentage of larvae that had settled or metamorphosed after 24 h was analyzed using a chi-squared goodness-of-fit test, a sample statistic based on discrete frequencies (Sokal and Rohlf, 1995; pp. 695–696).

Larval response to extracts of Delisea pulchra *and* Ecklonia radiata

We investigated chemical cues from algae by examining whether larvae of *H. purpurascens* would preferentially settle or metamorphose in response to metabolites extracted from its host algae *D. pulchra* and *E. radiata*. Both water-soluble (polar) and lipid-soluble (nonpolar) extracts of the algae were investigated. To prepare extracts, each alga (~10 g wet weight) was collected from Bare Island and blotted dry, epiphytes and epifauna were removed, and the thallus was exhaustively extracted in methanol (MeOH). The extracts were filtered and reduced *in vacuo* at 50°C prior to partitioning between water (MilliQ) and dichloromethane (DCM). The concentrated polar extract of each alga was prepared by reducing the water-soluble fraction *in vacuo* to a volume of 10 ml. The nonpolar extract of each alga was prepared by reducing the DCM-soluble fraction to dryness *in vacuo*.

The polar extracts from *D. pulchra* were tested at final concentrations of 10 μl and 1 μl ml^{-1} in 5 ml of seawater, and the nonpolar extracts were tested at 1 μg ml^{-1} and 100 ng ml^{-1} in 5 ml of sterile filtered seawater (higher concentrations were not tested because they caused abnormal behavior in the larvae). The polar and nonpolar extracts of *E. radiata* were tested in 5 ml of sterile filtered seawater at the highest concentrations of 10 μl ml^{-1} and 1 μg ml^{-1} respectively. Solutions of crude nonpolar extract were made by dissolving the extract in dimethylsulfoxide (DMSO; 60 μl mg^{-1} of extract) and diluting to the above concentrations in sterile filtered seawater. The maximum concentration of DMSO in the extracts was 0.006%, and DMSO at this concentration was used as a control in the assays for the nonpolar extracts. MilliQ water (10 μl ml^{-1}) was included as a control for the assays with polar extracts, and sterile filtered seawater was used as a further control for both extracts. *D. pulchra* (15 mg per replicate) was included as a treatment to ensure that the larvae were competent to metamorphose (a "positive" control). One competent larva of *H. purpurascens* was added to each replicate petri dish (n = 10 dishes per treatment) and the percent settlement and metamorphosis of larvae was recorded over time. Data after 20 h were analyzed with chi-squared goodness-of-fit tests as before.

Isolation and identification of the settlement inducer from Delisea pulchra

To isolate and characterize the active inducer, *D. pulchra* (~1 kg wet weight) was collected from Bare Island, and the extract was prepared as above. The polar and nonpolar phases were separated, and the water fraction was retained and evaporated to dryness *in vacuo*. The dried, crude polar extract was redissolved in absolute ethanol (AR) and re-dried. This process was repeated twice. Analysis by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy indicated that the resultant extract was predominantly composed of sugars. Fractionation of the extract by reversed-phase high-performance liquid chromatography (HPLC; Adsorbosil C18 @ 5 μm; 250 mm × 7.8 mm, Waters R410 RI detector) (100% MeOH, 2 ml min^{-1}) afforded two products: [1] and [2]. ^{1}H and ^{13}C NMR data and high field two-dimensional NMR studies (COSYDQF, HMBC and NOESY) (d_4 MeOH, Bruker DMX 500 NMR) were used to identify [1] and [2].

Larval response to isolated metabolites

The two products of the separation of the polar extract—[1] and [2]—were made up as stock solutions in MilliQ water, and aliquots were added to assay dishes to give final concentrations of 25 μ*M* and 2.5 μ*M*. MilliQ water (10 μl ml^{-1}) and sterile filtered seawater were used as controls. *D. pulchra* (15 mg per replicate) was included as a treatment as a measure of the competency of the larvae. One larva of *H. purpurascens* was added to each replicate petri dish (n = 12 dishes per treatment), and the percent metamorphosis of larvae was recorded over time. Metamorphosis after 5 h was analyzed with a chi-squared goodness-of-fit test as before.

Larval response to isolated and synthetic inducers

To confirm the activity of the floridoside-isethionic acid complex [1] as an inducer of metamorphosis, a synthetic floridoside–isethionic acid complex was prepared. Floridoside [α-D-galactopyranosyl-(1-2)-glycerol] was isolated following a modification of the method of Karsten *et al.* (1993). *D. pulchra* was collected from Bare Island and oven dried at 80°C. The dried tissue (179.4 g) was extracted with 1 l of 70% ethanol (v/v) overnight in a water bath at 70°C. The extract was reduced *in vacuo* and partitioned between water and ethyl acetate. The aqueous phase was reduced *in vacuo*, redissolved in absolute ethanol (AR), and subsequently taken to dryness. This residue was redissolved in MilliQ water (100 ml) and passed through a column of AG50WX2 (H^+ form) ion-exchange resin (Bio-Rad). Four fractions of 25 ml each were collected and analyzed by ^{1}H and ^{13}C NMR spectroscopy. Subsequently, the first two fractions were passed through a column of AG1X8 (OH^- form) ion-exchange resins (Bio-Rad). The eluted fractions were combined and evaporated to dryness *in vacuo* to yield floridoside as colorless prisms. The ^{1}H and ^{13}C NMR data for floridoside agreed with the reported spectroscopic data (Karsten *et al.*, 1993). Isethionic acid was prepared by passing a solution of sodium isethionate (Aldrich) in MilliQ water through a column of AG50WX2 (H^+ form) ion-exchange resin. Purity was confirmed by comparison of ^{1}H and ^{13}C NMR data (Barrow *et al.*, 1993). Equimolar amounts of floridoside and isethionic acid were mixed in a reacti-vial and allowed to stand in a water bath overnight at 55°C prior to being used in the bioassay.

All compounds—[1], the synthetic floridoside–isethionic acid complex, floridoside [2], and isethionic acid—were made up in 10 mg ml^{-1} stock solutions in MilliQ water, and aliquots were added to treatment dishes to give a final concentration in the bioassay of 200 μ*M*. MilliQ water and seawater controls were incorporated in the assay. *D. pulchra* (15 mg per replicate) was used as a positive measure of competency of the larvae. For the assay, one larva of *H. purpurascens* was added to each replicate petri dish (n = 10 dishes per treatment), and the percent metamorphosis of larvae was recorded over time. Data were analyzed with chi-squared goodness-of-fit tests as before.

Concentration-dependent response of larvae to the floridoside-isethionic acid complex

The response of competent larvae of *H. purpurascens* to a concentration gradient of [1] was tested to determine the minimum concentration for the induction of metamorphosis. [1] was tested at six concentrations: 125, 62.5, 25, 12.5, 2.5, and 1.25 μ*M*. The complex [1] was made up in a 10 mg ml^{-1} stock solution in MilliQ water, and aliquots were added to treatment dishes with 0.45-μm filtered seawater to give the six concentrations in the assay. MilliQ water and seawater controls also were incorporated as treatments (*i.e.*, controls) in the assay. *D. pulchra* (15 mg per replicate) was again used as a positive measure of competency of the larvae. One larva of *H. purpurascens* was added to each replicate petri dish (n = 12 dishes per treatment), and the percent metamorphosis of larvae recorded over time. Metamorphosis after 6 h was analyzed with chi-squared goodness-of-fit tests as before.

Larval response to conditioned seawater collected in situ

Seawater samples were collected from Bare Island on a calm, sunny November afternoon to test for the *in situ* presence of a waterborne cue associated with *D. pulchra*. Plastic syringes were used to collect seawater from within 0.5 cm of fronds of *D. pulchra* and laminae of *E. radiata* but without touching the plants, and from the water column more than 2 m distant from any macroalgae. Samples (5 ml) were immediately transferred into glass vials and stored in the dark until the start of the experiment (~3 h later). Each

sample was added to a separate petri dish (n = 10 dishes per treatment), along with one competent larva of *H. purpurascens*. Sterile filtered seawater controls were used in the assay as controls, and the percent metamorphosis of larvae was recorded over time.

Larval response to subtidal algae

Two replicate assays were done with *H. purpurascens* to determine whether larvae metamorphose in response to associative cues from other local species of algae, including a range of red algae. Ten species of subtidal algae, including the two common host plants of *H. purpurascens*, were collected from Bare Island on two occasions (November 1997 and February 1998), including six species of red algae: *Delisea pulchra, Laurencia rigida, Solieria robusta, Corallina officinalis, Amphiroa anceps, Pterocladiella capillacea;* two species of brown algae: *Ecklonia radiata, Sargassum linearifolium;* and two species of green algae: *Caulerpa filiformis* and *Ulva* sp. For each collection, the algae were brought back to the laboratory and 15 mg of the tip of each plant that was free of epiphytes and epifauna was removed and used in the assay. Each replicate (n = 12 dishes per treatment) consisted of the tip of one type of alga in 5 ml of sterilized filtered seawater in a glass petri dish. The response of larvae to sterile filtered seawater was used as a control. One competent larva of *H. purpurascens* was added to each replicate petri dish, and the percent metamorphosis of larvae was recorded after 24 h. For species of algae that induced metamorphosis, data comparing dates of collection were analyzed with a chi-squared goodness-of-fit test as before.

Results

Size distribution and abundance of Holopneustes purpurascens *on host plants*

The size distribution of *Holopneustes purpurascens* on its two main hosts differed significantly (Fig. 1). Of the 272 urchins found on *Delisea pulchra* and the 276 urchins on *Ecklonia radiata*, those inhabiting *D. pulchra* were significantly smaller than those found on *E. radiata* (unpaired t test, t = 4.30, $P < 0.001$). No urchins in the smallest size class (0–5 mm, reflecting new or recent recruits) were found on *E. radiata*, and small urchins in general (6–20 mm) were extremely rare on *E. radiata* (Fig. 1). Conversely, large urchins (>40 mm) were relatively scarce on *D. pulchra*, constituting only 5.1% of all observations made, compared to 73% of the total observations made on *E. radiata*. The abundance of *H. purpurascens* on *D. pulchra* and *E. radiata*, however, did not differ (one-factor ANOVA, $F_{1,38}$ = 2.023, P = 0.163; untransformed data).

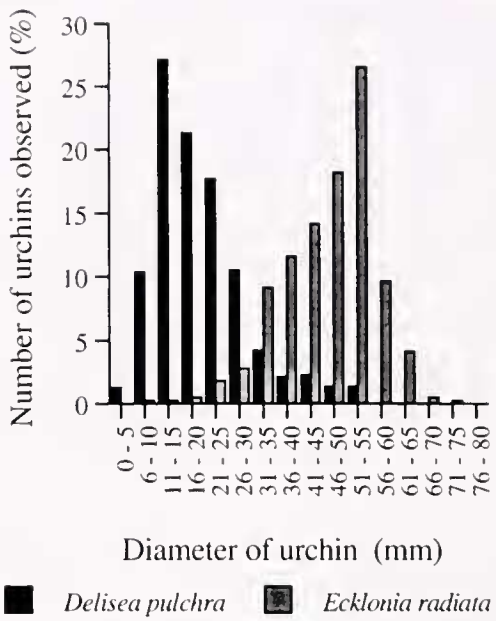

Figure 1. Size distributions of *Holopneustes purpurascens* on randomly selected plants of *Delisea pulchra* (number of urchins (n) = 272) and *Ecklonia radiata* (n = 276) at Bare Island, pooled over two seasons.

Larval response to conspecifics

No significant settlement or metamorphosis was observed after 24 h for competent larvae of *H. purpurascens* that were exposed to newly metamorphosed conspecifics or to water conditioned by adult conspecifics (X^2 = 4.68, 2 df, P = 0.086). No metamorphosis was observed in any of the treatments or controls, and of the 16 replicates, only one replicate larva had settled in the presence of one new recruit and two replicate larvae had settled in the presence of two new recruits. After 48 h, settlement was reduced to one individual in the presence of one new recruit. We concluded that settlement or metamorphosis in *H. purpurascens* was not aggregative, and subsequent assays were done with one larva per replicate petri dish.

Larval response to extracts of Delisea pulchra *and* Ecklonia radiata

There were significant differences in the response of larval *H. purpurascens* to the different extracts. All larvae that had been exposed to polar extracts from *D. pulchra* settled within the first 3 h of the experiment at both concentrations, whereas larvae exposed to pieces of *D. pulchra* settled after 7 h (Fig. 2A). These treatments had the highest final amount of settlement (Fig. 2A). Settlement of larvae exposed to nonpolar extracts from *D. pulchra* did occur, but this response was not constant over time and was significantly less than for polar treatments at the end of the assay (X^2 = 21.95, 3 df, $P < 0.001$). Although an initial settlement response occurred in the presence of pieces of *E.*

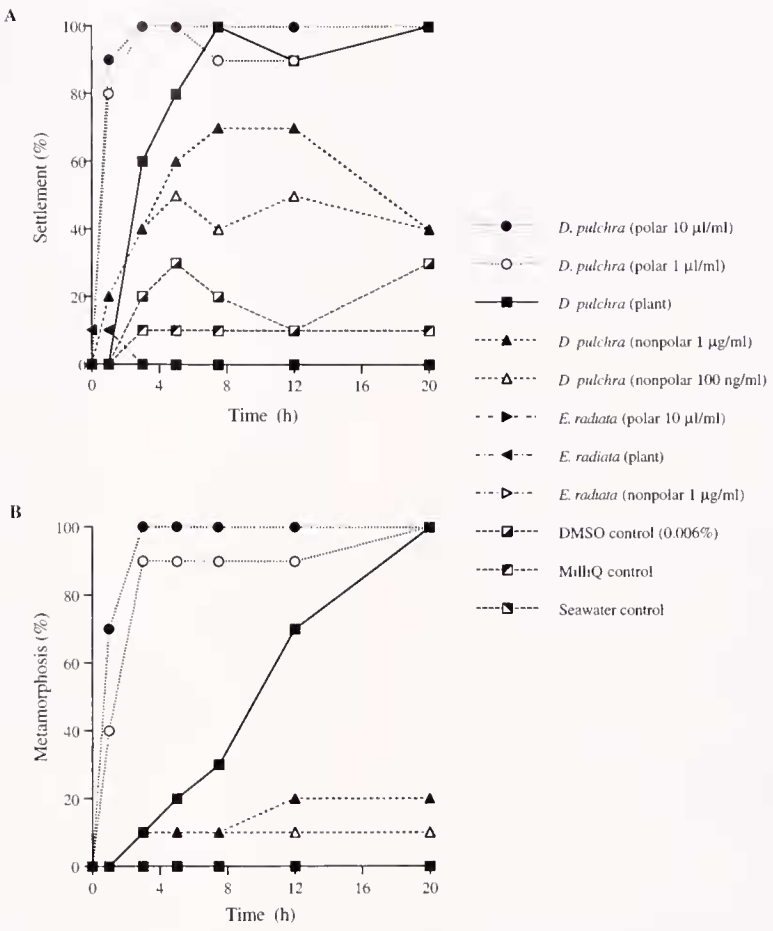

Figure 2. Percent (A) settlement and (B) metamorphosis over time of competent *Holopneustes purpurascens* (one larva per replicate; 10 replicates per treatment) when exposed to pieces of *Delisea pulchra* (15 mg per replicate) and its polar and nonpolar extract fractions. Controls of MilliQ water and sterile, filtered seawater were used.

radiata, no settlement occurred in response to its polar or nonpolar extracts. At the end of the assay, no significant difference in settlement was observed between larvae exposed to treatments containing *E. radiata*, and the MilliQ and seawater controls ($X^2 = 10.67$, 5 df, $P = 0.058$).

Larval *H. purpurascens* metamorphosed in response to extracts from *D. pulchra* but not those from *E. radiata* (Fig. 2B). After 20 h, all larvae of *H. purpurascens* had metamorphosed in the presence of pieces of *D. pulchra* and its polar extract, regardless of concentration (Fig. 2B). The polar extracts at both concentrations induced metamorphosis at a faster rate than did pieces of the alga. All larvae exposed to polar extracts from *D. pulchra* at 10 µl ml^{-1}, and the majority of larvae exposed to polar extracts from *D. pulchra* at 1 µl ml^{-1}, metamorphosed within 3 h (Fig. 2B). Nonpolar extracts from *D. pulchra* also induced a low percentage of metamorphosis; however, this rate was significantly lower than that for both polar extracts (20 h: $X^2 = 38.04$, 3 df, $P < 0.001$; Fig. 2B) and may have been due to small residual amounts of polar metabolites remaining in these nonpolar extracts. No metamorphosis occurred in the controls.

Isolation and identification of the settlement inducer from Delisea pulchra

The polar fraction of *D. pulchra* yielded two metabolites [1] and [2] (Fig. 3). The ^1H and ^{13}C NMR data for [1] corresponded to previously published data for floridoside [α-D-galactopyranosyl-(1-2)-glycerol] (Karsten *et al.*, 1993) and isethionic acid (Barrow *et al.*, 1993) in 1:1 integral ratios (Fig. 3). Spectroscopic data for [2] corresponded to floridoside [α-D-galactopyranosyl-(1-2)-glycerol] (Fig. 3). We were unable to further separate [1] using standard chromatographic techniques. High field two-dimensional NMR studies (COSYDQF, HMBC and NOESY) of [1] showed correlations between the proton and carbon signals identified as those for floridoside and isethionic acid respec-

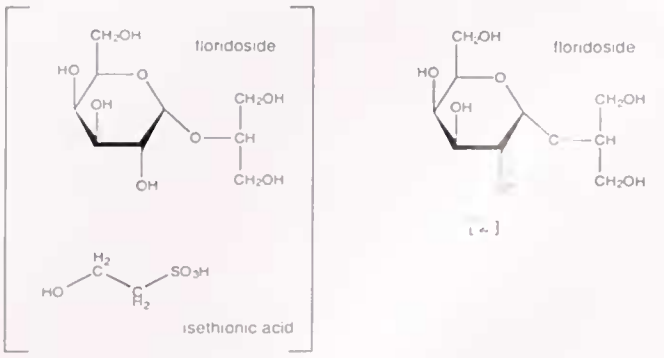

Figure 3. Structures of the floridoside and isethionic acid complex [1] and of floridoside [2].

tively within the spectra for [1]; however, there were no correlations between signals for the two compounds. Furthermore, floridoside and isethionic acid were separately spiked into [1] in d_4 MeOH, and the mixture was analyzed by ^{13}C NMR spectroscopy before and after spiking. Addition of floridoside and isethionic acid resulted in an increase in their respective signals in the ^{13}C NMR spectra of [1]. No additional signals were detected. This demonstrates the absence of any covalent bonding between the two species and is consistent with [1] being a complex of floridoside and isethionic acid (Fig. 3).

Larval response to isolated metabolites

Of the two metabolites [1] and [2], only the floridoside–isethionic acid complex [1] induced metamorphosis in larvae of *H. purpurascens* (Fig. 4). Therefore, we concluded that the active cue for metamorphosis of *H. purpurascens* was the floridoside–isethionic acid complex [1], as isolated from the polar extract of *D. pulchra* (Fig. 4). Floridoside [2] did not induce any metamorphosis of *H. purpurascens* after 15 h. The rate of metamorphosis of *H. purpurascens* in response to the floridoside–isethionic acid complex [1] was concentration dependent, with larvae exposed to 25 μM metamorphosing significantly faster than those exposed to 2.5 μM (5 h: $X^2 = 21.02$, 1 df, $P < 0.001$; Fig. 4). The higher concentration of 25 μM of [1] induced more rapid metamorphosis than did pieces of *D. pulchra* (Fig. 4). No metamorphosis was observed in either of the controls.

Larval response to isolated and synthetic inducers

The activity of the floridoside–isethionic acid complex was confirmed in assays comparing the natural and synthetic inducer. Larvae of *H. purpurascens* metamorphosed in a similar manner in the presence of both natural and synthetic complexes of the floridoside-isethionic acid complex (Fig. 5). After 24 h, substantially more metamorphosis occurred in treatments of pieces of *D. pulchra*, the pure complex [1] as isolated from the crude polar extract (200 μM), or synthetic floridoside–isethionic acid (200 μM) than in other treatments ($X^2 = 57.15$, 6 df, $P < 0.001$; Fig. 5). The synthetic floridoside–isethionic acid complex mimicked the percentage of metamorphosis of the natural floridoside–isethionic acid product [1] (Fig. 5). No metamorphosis was observed in treatments containing floridoside or isethionic acid, or in the controls. There was no significant change in the percentage of *H. purpurascens* that had meta-

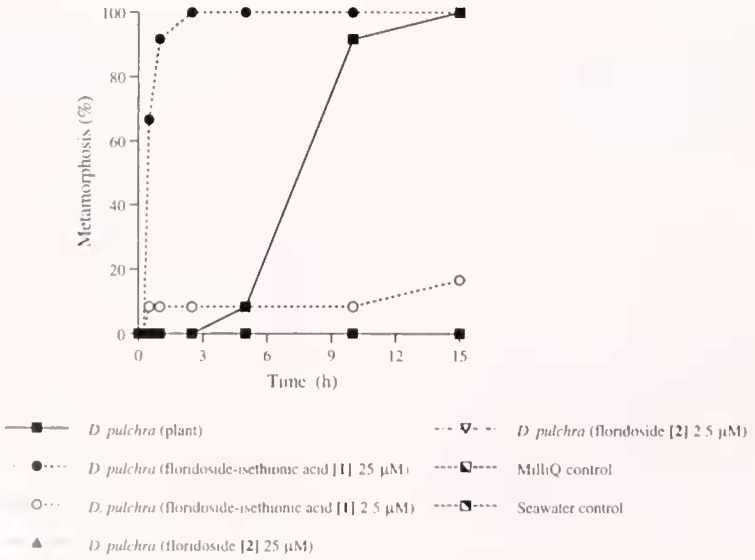

Figure 4. Percent metamorphosis over time of competent larvae of *Holopneustes purpurascens* (one larva per replicate, 12 replicates per treatment) when exposed to pieces of *Delisea pulchra* (15 mg per replicate) and the floridoside–isethionic acid complex [1] at two concentrations. Controls of MilliQ water and sterile, filtered seawater were used.

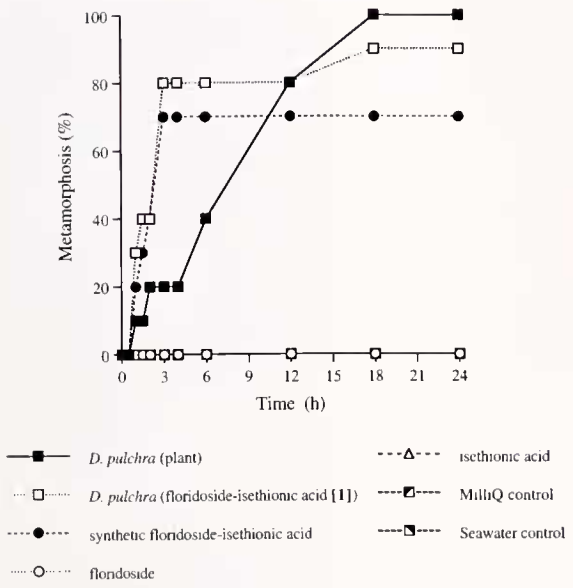

Figure 5. Percent metamorphosis over time of competent larvae of *Holopneustes purpurascens* (one larva per replicate; 10 replicates per treatment) when exposed to pieces of *Delisea pulchra* (15 mg per replicate), or 200 μM of either [1] as isolated from the crude polar extract of *D. pulchra*, the synthetic floridoside-isethionic acid complex in a 1:1 molar ratio, floridoside, or isethionic acid. Controls of MilliQ water and sterile, filtered seawater were used.

morphosed from 24 to 36 h, at which point the assay was stopped ($X^2 > 0.05$, 2 df, $P = 0.978$).

Concentration-dependent response of larvae to the floridoside-isethionic acid complex

The larvae of *H. purpurascens* metamorphosed at a concentration-dependent rate when exposed to different levels of the floridoside–isethionic acid complex [1] (Fig. 6). Larvae metamorphosed at a faster rate at higher concentrations of [1], with all concentrations from 125 μM to 25 μM inducing metamorphosis of larvae at the same rate. These concentrations also induced metamorphosis more rapidly than did pieces of *D. pulchra* (6 h: $X^2 = 24.88$, 3 d.f., $P < 0.001$). Concentrations of 12.5 μM and 2.5 μM also induced metamorphosis, albeit significantly more slowly than higher concentrations (6 h: $X^2 = 36.39$, 4 df, $P < 0.001$), and no metamorphosis occurred at a concentration of 1.25 μM, or in controls (Fig. 6).

Larval response to conditioned seawater collected in situ

Seawater surrounding *D. pulchra* induced 100% metamorphosis of larvae at a rate comparable to that of the floridoside-isethionic acid complex in earlier assays (compare Fig. 5 to Fig. 7). Seawater surrounding *E. radiata*, or water sampled away (>2 m) from any benthic algae, did not induce metamorphosis (Fig. 7).

Larval response to subtidal algae

As well as responding to *D. pulchra*, larvae of *H. purpurascens* were induced to metamorphose within 24 h in the presence of four other species of red algae (*Laurencia rigida*, *Solieria robusta*, *Corallina officinalis*, and *Amphiroa anceps*) in varying degrees (Fig. 8). *D. pulchra* induced the greatest response, with all larvae exposed to this alga metamorphosing (Fig. 8). Most of the larvae exposed to *L. rigida* also metamorphosed after 24 h. Larval response to the three other red algae was lower and more varied, and no metamorphosis occurred in the presence of any of the brown or green algae tested (Fig. 8). This pattern in the proportion of metamorphosis for algal species was consistent between the two times analyzed ($X^2 = 5.86$, 1 df, $P = 0.209$).

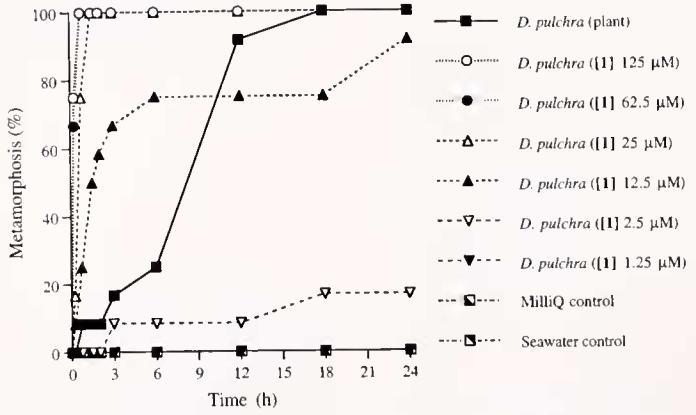

Figure 6. Percent metamorphosis over time of competent larvae of *Holopneustes purpurascens* (one larva per replicate; 12 replicates per treatment) when exposed to pieces of *Delisea pulchra* (15 mg per replicate) and different concentrations of the floridoside-isethionic acid complex. Controls of MilliQ water and sterile, filtered seawater were used.

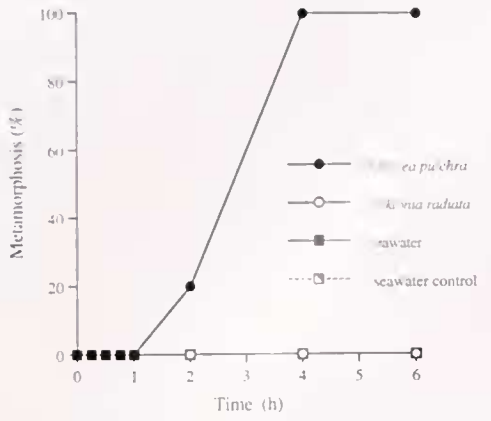

Figure 7. Percent metamorphosis over time of competent larvae of *Holopneustes purpurascens* (one larva per replicate; 10 replicates per treatment) when exposed to seawater surrounding plants of *Delisea pulchra* and *Ecklonia radiata*, and from seawater distant (>2 m) from any macroalgae. Sterile filtered seawater was used as a control.

Discussion

Environmental cues—physical, biological, or chemical—have long been thought to play an important role in the settlement and metamorphosis of marine invertebrate larvae (Svane and Young, 1989; Pawlik, 1992; McEdwards, 1995), and chemical cues in particular have received considerable attention in the last 20 years. Such chemical cues may be waterborne, associated with the surfaces of substrata, or derived from conspecifics (Pawlik, 1992), and there is now a substantial list of metabolites known to have strong effects on the settlement or metamorphosis of invertebrate larvae (reviewed in Pawlik, 1992; Slattery, 1997).

Few if any of these metabolites, however, have been characterized, quantified *in situ*, and shown to affect settlement or metamorphosis of larvae in a way that is consistent with the demography of the target organisms in the field. Many studies in this area have focused on what Pawlik (1992) termed "artificial" inducers; that is, the metabolites have not been shown to be present (either at all, or in active levels) in the environment of the settling organism, but rather are thought to mimic the action of naturally occurring inducers. Examples include the neurotransmitters γ-aminobutyric acid (GABA) (Morse *et al.*, 1979), L-β-3,4-dihydroxyphenylalanine (L-DOPA) (Coon and Bonar, 1987), and epinephrine (Coon *et al.*, 1986). Such compounds, many of which are widely distributed throughout the animal phyla, may play important internal roles as mediators of settlement and metamorphosis (see review in Coll *et al.*, 1989). Other [illegible] have identified naturally occurring inducers or [illegible] are produced in the environment exogenously to the [illegible] but they have either failed to characterize (and [illegible]tify) the relevant compound or have been unable to [illegible] the production or presence of the cue to the recruitment pattern of the organism (*e.g.*, Yvin *et al.*, 1985; Boettcher and Targett, 1996). Despite considerable ongoing work in this area (*e.g.*, Morse and Morse, 1984; Hadfield and Scheuer, 1985; Burke, 1986; Pawlik, 1986, 1988; Cochard *et al.*, 1989; Jensen and Morse, 1990; Morse, 1992; Davis and Stoner, 1994; Zimmer-Faust and Tamburri, 1994; Walters *et al.*, 1996; Zimmer-Faust *et al.*, 1997; Daume *et al.*, 1999; Tsukamoto *et al.*, 1999), to our knowledge there is no known chemical cue for larval settlement or metamorphosis that (a) can be clearly related to the pattern of settlement and recruitment of the target organism in the field, (b) is structurally characterized, and (c) is known to be present *in situ* at active concentrations.

We have demonstrated that the floridoside–isethionic acid complex fulfills many of the criteria necessary for an ecologically relevant cue for larval metamorphosis. The echinoid *Holopneustes purpurascens* was found almost exclusively on two host plants at our study site, *Delisea pulchra* and *Ecklonia radiata*. Although the abundance of urchins inhabiting the two hosts did not differ, the size of urchins on *D. pulchra* was significantly smaller than those on *E. radiata*, and in particular the smallest urchins (0–5 mm, reflecting new or recent recruits) were found exclusively on *D. pulchra*. Settlement or metamorphosis of larvae of *H. purpurascens* was not gregarious, unlike that of a

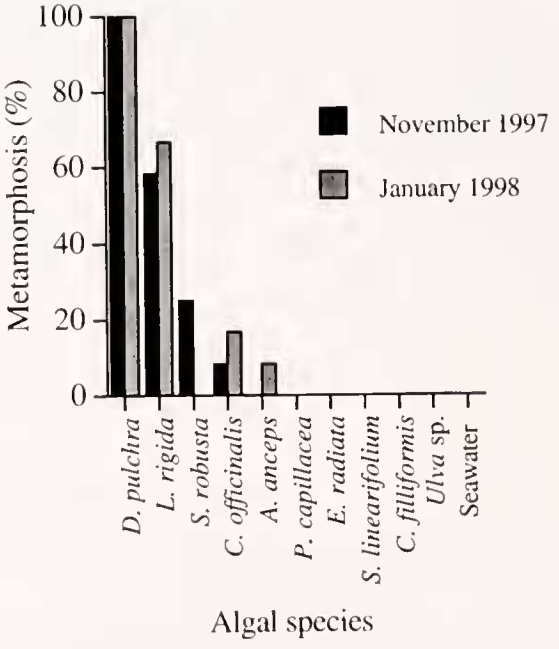

Figure 8. Percent metamorphosis of competent larvae of *Holopneustes purpurascens* (one larva per replicate; 12 replicates per treatment) after 24 h when exposed to subtidal algae (15 mg per replicate) from Bare Island on two occasions. Treatments included six species of red algae: *Delisea pulchra, Laurencia rigida, Soliera robusta, Corallina officinalis, Amphiroa anceps, Pterocladiella capillacea;* two species of brown algae: *Ecklonia radiata* and *Sargassum linearifolium;* and two species of green algae: *Caulerpa filiformis* and *Ulva* sp. The response of larvae to sterile filtered seawater was used as a control.

number of other echinoderms (Highsmith, 1982; Young and Chia, 1982; Pearce and Scheibling, 1990) and other invertebrates (Burke, 1986). Rather, these larvae metamorphosed in the presence of *D. pulchra*, the host on which new or recent recruits were found in the field. They showed no response to the alternative host, *E. radiata*, or its extracts. The larvae did not need to touch *D. pulchra* to metamorphose, indicating that the cue was water soluble.

Through bioassay-guided fractionation, the cue for metamorphosis was demonstrated to be a noncovalently-bound complex of the sugar floridoside and isethionic acid. The unusual (complexed) nature of the cue was confirmed by NMR spectroscopy, and by showing that the two components separately had no effect on metamorphosis. Larvae responded to the floridoside–isethionic acid complex in a dose-dependent manner, at concentrations similar to those of active levels of previously described inducers. Their response to synthetically prepared floridoside–isethionic acid complex was similar to their response to the naturally extracted cue.

As with all previous studies of larval inducers, we have not yet shown that the floridoside–isethionic acid complex is present *in situ* at levels that induce metamorphosis of *H. purpurascens*. We are currently developing quantitative methods in our laboratory, as well as methods for experimentally deploying the cue in the field (analogous to the "larval flypaper" of Morse and Morse, 1986). However, also consistent with the hypothesis that the floridoside–isethionic acid complex is an *in situ* cue, larvae metamorphosed in the presence of water collected close to *D. pulchra*, but not in the presence of water collected adjacent to *E. radiata* or away (>2 m) from any macroalgae. Larvae also metamorphosed in the presence of other red, but not green or brown, algae. Although other cues may be present in these algae, this is further support for the induction of metamorphosis by the floridoside–isethionic acid complex, as floridoside is found widely in red algae (Karsten *et al.*, 1993), but not in brown or green algae. These other red algae are less common than *D. pulchra* in our study habitats, and *H. purpurascens* is rarely found on these other species. One explanation for this pattern is that the greater abundance of *D. pulchra* may simply result in higher local concentrations of a shared cue (such as the floridoside–isethionic acid complex) in water near beds of *D. pulchra* than in water surrounding other local species of red algae. Concentrations of the metabolites may also vary among the different species, as is well known for both primary (*e.g.*, floridoside; Karsten *et al.*, 1999) and secondary (Hay, 1996) algal metabolites.

The response of *H. purpurascens* to red, but not green, algae is consistent with numerous previous studies indicating that sea urchins often settle or metamorphose preferentially in response to red algae, particularly corallines. For example, the sea urchins *Pseudocentrotus depressus* and *Anthocidaris crassispina* preferentially metamorphose in response to the coralline alga *Corallina pilulifera* (Kitamura *et al.*, 1993, 1994). Similarly, species of *Strongylocentrotus* preferentially metamorphose in response to turfing and crustose red algae such as *C. officinalis, Lithothamnion glaciale, Phymatolithon laevigatum,* and *P. rugulosum* (Hart and Scheibling, 1988; Pearce and Schiebling, 1991), and to a partially purified extract of coralline algae (Rowley, 1989). One interesting difference between the responses of *H. purpurascens* and other urchins cited above is that *H. purpurascens* did not respond most strongly to coralline algae. The four other species of red algae tested, including the corallines *C. officinalis* and *Amphiroa anceps*, also all induced metamorphosis but to a lesser degree than *Delisea pulchra*.

Red algae are also thought to be the primary stimuli for induction of settlement or metamorphosis among other invertebrates, including the coral *Agaricia humilis* (Morse, 1992), the abalone *Haliotis rufescens* (Morse *et al.*, 1979) and *H. laevigata* (Daume *et al.*, 1999), the queen conch *Strombus gigas* (Davis and Stoner, 1994; Boettcher and Targett, 1996), the sea hare *Aplysia californica* (Nadeau *et al.*, 1989), and the scallop *Pecten maximus* (Cochard *et al.*, 1989).

Although settlement in response to red algae is common, the cues responsible for these responses are largely unknown. Only one previous study has identified a naturally produced inducer from red algae, jacaranone, isolated from aqueous extracts of the red alga *Delesseria sanguinea* (Yvin *et al.*, 1985). However, although jacaronone stimulated metamorphosis of the bivalve scallop *Pecten maximus*, co-occurrence of the alga and the scallop was not reported, and subsequent reports indicated that these scallops lacked "substrate-specific recruitment" (Cochard *et al.*,1989). Perhaps the best known example of red algal cues is that of Morse and co-workers, who showed that larvae of the abalone *Haliotis rufescens* settled and metamorphosed preferentially onto red crustose algae such as *Lithothamnion* sp., *Lithophyllum* sp., and *Hildenbrandia* sp. (Morse *et al.*, 1979). Larvae of *H. rufescens* were also induced to settle and initiate metamorphosis in response to GABA. Morse and Morse (1984) subsequently found a mimetic peptide of GABA within the surface of the crustose red algae, which they concluded was likely to be the active cue for settlement and metamorphosis of *H. rufescens*. However, the mimetic peptide has not to date been characterized (research on the role of GABA and its mimetics in induction of larval settlement is reviewed by Slattery, 1997).

A recurring theme in the literature (see reviews by Pawlik, 1992; Slattery, 1997) on induction of larval settlement is whether cues are primarily nonpolar substances that are adsorbed to or incorporated within substrata, requiring larvae to physically contact a surface before encountering the cue (*e.g.*, Pawlik and Faulkner, 1986; Morse *et al.*, 1994; Clare and Matsumura, 2000), or whether they are polar

metabolites that are detected in the water column, such as the cue or cues for *H. purpurascens*. Although both surface adsorbed (nonpolar) and water-soluble cues may be important for some larvae (Clare and Matsumura, 2000), a number of studies have highlighted the importance of waterborne, low-molecular-weight, water-soluble cues for settlement or metamorphosis of a taxonomically diverse array of invertebrates. These include the upside-down jellyfish *Cassiopea xamachana* (Fleck and Fitt, 1999), the tube worm *Hydroides elegans* (Walters *et al.*, 1996), the red abalone *Haliotis rufescens* (Morse *et al.*, 1984; Barlow, 1990), the queen conch *Strombus gigas* (Boettcher and Targett, 1996), the nudibranch *Phestilla sibogae* (Hadfield and Scheuer, 1985), the oyster *Crassostrea virginica* (Turner *et al.*, 1994; Zimmer-Faust and Tamburri, 1994; Browne *et al.*, 1998), and the Pacific sand dollar *Dendraster excentricus* (Burke, 1984). This pattern contrasts with the suggestion that naturally produced deterrents of settlement ("natural antifoulants") are likely to be nonpolar and thus able to adsorb to the surface of the producing organism (Schmitt *et al.*, 1995; Jennings and Steinberg, 1997; Steinberg *et al.*, 1998; Dworjanyn *et al.*, 1999).

One of the major arguments against the effectiveness of waterborne cues for induction of settlement is that *in situ* hydrodynamics will weaken the effect of the cue to the point where it ceases to be an effective signal. This can happen both by currents or turbulence inhibiting weakly swimming larvae from reaching an appropriate habitat once the cue has been detected (Chia *et al.*, 1984; Butman, 1986), or by the surrounding seawater simply diluting the cue to a level below larval detection. This assumed dilution and rapid dispersion of water-soluble chemicals in flowing seawater has resulted in such substances being traditionally dismissed as potential cues (Butman, 1989). Indirect support for this argument also comes from data showing that for some invertebrates, larval delivery to sites is similar to what would be expected for the delivery of inert particles with similar characteristics (*e.g.*, Eckman, 1983; Mullineaux and Butman, 1991; but see Eckman and Duggins, 1998).

In contrast to the "dilution" or "weakly swimming" hypotheses, several studies suggest that water-soluble cues can function as *in situ* inducers of settlement or metamorphosis. We have shown that larvae of *Holopneustes purpurascens* metamorphosed in response to seawater surrounding *Delisea pulchra* collected *in situ*, indicating that larvae of this urchin do detect and respond to *in situ* concentrations of a water-soluble inducer. Tamburri *et al.* (1996) showed that larvae of the oyster *Crassostrea virginica*, known to be weak swimmers, consistently detected and responded to waterborne chemical cues in raceway flumes at flow speeds and shear velocities typical of estuarine flows. In both of these studies, response of the larvae to the presence of a cue was very rapid. *C. virginica* responded within 5 s of initial exposure to the waterborne cue (Tamburri *et al.*, 1996). As with the floridoside–isethionic acid complex, larvae of *H. purpurascens* in still-water assays rapidly changed their position in the water column, moving to the bottom of petri dishes within 5 min of exposure to the cue (Williamson, pers. obs.). Within 15 min of exposure, the majority of larvae had settled and begun to metamorphose, suggesting that these larvae were able to rapidly respond both behaviorally and developmentally within a short time of detecting the cue. A rapid response to a cue, either behaviorally or developmentally (through metamorphosis), would obviously facilitate the ability of larvae to respond effectively to waterborne cues from particular sources in their habitats.

As well as a rapid response time, several other parameters should affect the utility of water-soluble metabolites as cues for settlement or metamorphosis. Decho *et al.* (1998) have argued that basic (alkaline) peptides should be common as signal molecules in marine systems because of their ability to resist biodegradation by marine bacteria (as well as because of the general importance of small peptides as chemical signals for animal sensory systems). An additional likely criterion for an effective water-soluble cue is that it be produced in large amounts, so that the cue is maintained at an effective concentration near the surface from which it emanates. Both oyster beds (Tamburri *et al.*, 1992; Zimmer-Faust *et al.*, 1997) and the algal system described here would appear to meet this criterion. Although the oysters are perhaps an extreme example, many other invertebrates on either soft or hard substrata also occur in high-density, nearly monocultural aggregations (*e.g.*, Woodin, 1976). Such assemblages are likely places to look for realistic waterborne settlement or metamorphosis cues.

In particular, many shallow, hard-substratum communities are dominated by dense assemblages composed of one or a few species of benthic algae that provide habitat, and often food, for a diverse array of invertebrates. Benthic algae are well known to "leak" metabolites, particularly carbohydrates, from their thalli (Lobban and Harrison, 1994). Such macroalgal sugars are often produced in high concentrations (*e.g.*, Karsten *et al.*, 1993, for floridoside), are exuded from the thallus (Moebus and Johnson, 1974), and are in a number of cases unique to a particular algal group. For example, floridoside and isethionic acid are widespread (if not ubiquitous) among red algae, but are also unique to this algal group (Barrow *et al.*, 1993, 1995; Clayton and King, 1996). Similarly, mannitol and its derivatives are widespread within the brown algae, but do not occur in other seaweeds (Clayton and King, 1996). Algal sugars or sugar derivatives would thus seem to generally meet at least two important criteria for an effective water-soluble cue: (i) the potential to reach high concentrations *in situ*, and (ii) some degree of source (substratum) specificity. There are now several studies demonstrating or implicating algal sugars as larval settlement cues (Bahamondes-Rojas and Dherbomez, 1990; Boettcher and Targett, 1996).

Conclusion

We have shown that larvae of the echinoid *Holopneustes purpurascens* metamorphose in response to a chemical cue—composed of a complex of the red algal sugar floridoside and isethionic acid—from one of its two main host plants, the red alga *Delisea pulchra*. The presence of the cue in *D. pulchra*, but not the alternative host *Ecklonia radiata*, is consistent with the demography of *H. purpurascens*, in which the most recently recruited individuals are found only on *D. pulchra*. We are currently developing methods for the quantification of the floridoside–isethionic acid complex in the field, and for the controlled, experimental release of the cue *in situ* to fully test the hypothesis that the floridoside–isethionic acid complex is an ecologically relevant cue for the induction of metamorphosis of *H. purpurascens*. Such experiments would also enable us to test more generally the relative importance of hydrodynamics *versus* chemical cues for settlement and metamorphosis of larvae of *H. purpurascens*, and to understand the differential response of larvae to different species of red algae. Finally, in contrast to some previous suggestions, we suggest that water-soluble metabolites, such as algal sugars or their derivatives, may be generally important as cues for marine invertebrate settlement.

Acknowledgments

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